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X-Ray studies reveal lanthanide binding sites at the A/B₅ interface of *E. coli* heat labile enterotoxin

Titia K. Sixma, Anke C. Terwisscha van Scheltinga, Kor H. Kalk, Kangjing Zhou*, Ellen S. Wartna and Wim G.J. Hol

BIOSON Research Institute, Department of Chemistry, University of Groningen, Nijenborgh 4, 9747 AG Groningen, The Netherlands

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The crystal structure determination of heat labile enterotoxin (LT) bound to two different lanthanide ions, erbium and samarium, revealed two distinct ion binding sites in the interface of the A subunit and the B pentamer of the toxin. One of the interface sites is conserved in the very similar cholera toxin sequence. These sites may be potential calcium binding sites. Erbium and samarium binding causes a change in the structure of LT: a rotation of the A1 subunit of up to two degrees relative to the B pentamer.

Heat labile enterotoxin; Cholera toxin; Calcium; Lanthanide ion; Conformational change

1. INTRODUCTION

Heat labile enterotoxin (LT) from *E. coli* and the very similar cholera toxin (CT) consist of an AB₅ complex, in which the B pentamer is necessary for binding to the membrane of the target intestinal cell, to allow internalization of the enzymatic A subunit into the cytoplasm (for reviews see [1–3]). For activity this A subunit needs to be proteolytically nicked and reduced, yielding two fragments, called A1 (1–192) and A2 (~195–240). The A subunit acts by ADP-ribosylation of G_sα, which then remains in its activated state and causes production of elevated levels of cyclic AMP [4,5]. This has been indicated as the reason for the secretion of fluids and electrolytes caused by these toxins [6,7]. Other evidence shows that prostaglandins may be responsible for the increased efflux of ions and water by CT [8]. In addition, LT and CT have been shown to possess a calcium ionophore activity [9,10] as well as mitogenic effects [11]. Lanthanum ions have an inhibitory effect on the toxin induced secretion of Cl[−] and HCO₃[−] [12], as well on the calcium ionophore activity [13]. We present here X-ray structures of LT bound to two different ions from the lanthanide series. We have identified two distinct binding sites at the A/B interface

as well as a conformational change induced by this binding.

2. MATERIALS AND METHODS

Heat labile enterotoxin from porcine *E. coli* was purified and crystallised according to [14]. Crystals were transferred to a standard solution containing 0.175 M KF, 6% PEG 6000 (w/v) in 0.1 M Tris, 1 mM EDTA and 0.02% azide at pH 7.5 (buffer A). Native data were collected from crystals transferred to this solution. Lanthanide soaking was done after transfer of crystals to a solution of 0.175 M NaCl, 6% PEG 6000, 0.1 M Tris at pH 7.5 (buffer B), i.e. buffer A without EDTA and azide, and with NaCl instead of KF to avoid precipitation of fluoride salts. For samarium soaking buffer B was brought to pH 7.1, as a prevention against precipitation of samarium hydroxides, although some precipitate was still noticeable. Transfer from buffer A to buffer B or to the lower pH did not cause changes on precession photographs larger than those observed between native crystals in the standard buffer [15].

Precession photographs were collected of LT crystals in solutions of buffer B containing MgCl₂, CaCl₂, Sm(NO₃)₃, Gd₂(SO₄)₃ and Er(NO₃)₃ in concentrations ranging from 0.5 to 15 mM. For data collection, one crystal was soaked for two days in 2 mM Er(NO₃)₃. Another crystal was gradually transferred from 0.5 mM Sm(NO₃)₃ (one day) to 1.0 mM (one day) and then to 2.0 mM (2 h) before mounting. This crystal was slightly cracked as a result of this soaking procedure, but data collection to 3.4 Å could still be performed and was followed by taking a precession photograph.

Data were collected at room temperature on an Enraf Nonius FAST area detector system, using an Elliot GX 21 rotating anode as X-ray generator (Table 1). Processing was performed with MADNES [16]. Native data were treated with background plane fitting option and profile fitting with XDS [17]. Refinement of the native LT structure has been described elsewhere [18]. Er(NO₃)₃ and Sm(NO₃)₃ data were scaled to the native data set by local scaling procedures [19]. $|F_o(\text{lanthanide}) - F_o(\text{native})|$ Fouriers were calculated using model phases of the 2.3 Å refined native structure [18] and $(m|F_o(\text{native})| - D|F_c|) \sigma_A$ weights [20]. Rigid body refinement against the Er(NO₃)₃ and the Sm(NO₃)₃ data were performed using the program TNT [21], using the 2.3 Å native coordinates without water molecules as a starting model (Table 1).

Abbreviations: LT, heat labile enterotoxin; CT, cholera toxin; rms, root mean square; PEG, poly ethylene glycol.

Present address: Fujian Institute of Research on the Structure of Matter, Chinese Academy of Sciences, Fuzhou, Fujian, China.

Correspondence address: W.G.J. Hol, BIOSON Research Institute, Department of Chemistry, University of Groningen, Nijenborgh 4, 9747 AG Groningen, The Netherlands. Fax: (31) (50) 634800.

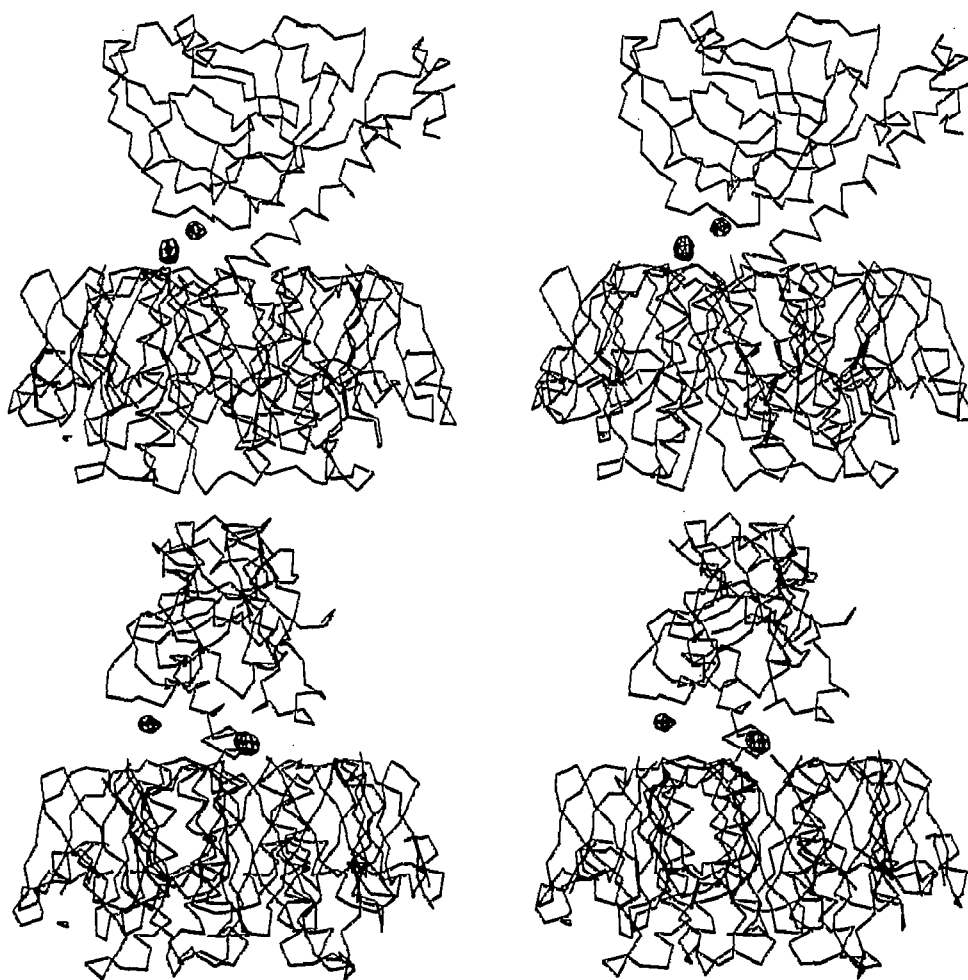


Fig. 1. Stereo figure showing the C α chain of LT AB₅ with difference electron density of three Sm³⁺ sites ($m|F_o(\text{Sm})| - D|F_c(\text{Sm})|$), phases $\alpha_c(\text{Sm})$, contoured at five sigma. Two sites are at the AB₅ interface, while the third (very weakly visible) is located in a crystal contact. Residues A:186–199 at the interface of A1 and A2 as well as A:235–240 at the C-terminus of A2 are not shown because of weak density. (A) The orientation is perpendicular to the 5-fold axis, with the A1 subunit on top of the B pentamer. (B) Same orientation as A but rotated 90° around the 5-fold axis.

3. RESULTS

Lanthanide soaking of heat labile enterotoxin crystals caused distinct cell dimension changes (Table I) as well as concentration-dependent intensity differences on precession photographs in comparison to native. Changes for erbium were smaller than for samarium and gadolinium, the latter two ions causing changes which were similar. Calcium, which has a similar ionic radius as the lanthanide ions but a different charge, did not cause large differences on precession photographs, at least not larger than those observed between native crystals [15], nor did magnesium. Data were collected of Sm(NO₃)₃ and Er(NO₃)₃ to a resolution of 3.4 Å. Rigid body refinement by subunit, and for Sm(NO₃)₃ with the A2 chain treated as two fragments, (residues 196–218 and 219–240) (see Table I), resulted in an overall shift of the molecule as well as a change in orienta-

tion of the A subunit with respect to the B pentamer. For erbium this rotation was 0.5°, for samarium 2.3°. These rotations of the A subunit with respect to the B subunits are quite remarkable since the lanthanide ions were introduced by the soaking procedure. Part of the A2 fragment (A2: 196–219) has made a similar rigid body movement as the A1 subunit, whereas the other half rotates together with the B pentamer.

Analysis of an $|F_o(\text{Er}) - F_c(\text{native})|$ difference Fourier revealed as highest peak one trivalent erbium binding site at the six sigma level. In the equivalent Sm(NO₃)₃ difference Fourier no sites could be identified, due to the larger changes in cell dimensions. After rigid body refinement, however, a $|F_o(\text{Sm}) - F_c(\text{Sm})|$ difference Fourier showed three distinct samarium binding sites at 8.5, 7.2 and 5.3 sigma levels of the map respectively (next highest peak at 4.7 sigma) (Fig. 1). The first site is equivalent to the erbium binding site, while the third



Fig. 2. Close up stereo figure of samarium sites in LT after coordinate refinement. The ligands of site no. 2, on the left side in the figure, are conserved in cholera toxin and may well be the site responsible for lanthanum inhibitor binding.

site is in a crystal contact between Glu B#5:11 and Glu A2:202 in two different molecules and will therefore not be discussed further.

The two ion binding sites are located in the A-B interface and have interactions with both A and B subunits (Figs. 1, 2). Due to the limited resolution of the present study no precise geometry of the ion environment can be given. Based on the positions of the side-chain carboxylates with respect to the ion sites ligands for site no. 1 are Glu-79 of B#5, Glu A1:144 and Asp A2:225 in the A2 chain. The second site involves Glu-79 in B#1, as well as Glu A1:29 and the C-terminus of B#5 (numbering of the subunits is according to [18]). Remarkably in both sites a B:Glu-79 residue is involved,

albeit from different subunits. In the remaining three B subunits this residue takes part in the A1-B interaction. From the A1 subunit the samarium ligands are located roughly within the two loops that form the only interaction with the B pentamer [18]. Samarium site no.1 (Fig. 2A) is not conserved in cholera toxin: both Glu A1:144 and Asp A2:225 in LT are glycines in CT, while only Glu B:79 is identical. The second ion binding site is well conserved: all three ligands are the same in LT and cholera toxin [22].

4. DISCUSSION

Heat labile enterotoxin is shown to bind samarium at

Table I
Data collection and refinement statistics

Dataset		Sm(NO ₃) ₃	Er(NO ₃) ₃	Native
Cell dimensions (Å)	<i>a</i> axis	118.8	119.3	119.2
(Space group P2 ₁ 2 ₁ 2 ₁)	<i>b</i> axis	97.5	97.8	98.2
	<i>c</i> axis	65.1	65.0	64.8
Resolution (Å)		3.4	3.4	2.3
Number of measurements		22,101	26,039	67,629
Number of unique reflections		5,779	5,889	29,769
<i>R</i> _{sym} on intensities (%)*		5.8	4.4	6.2
Completeness to 3.4 Å (%)		57.1	54.3	92.3
Completeness to 4.1 Å (%)		81.5	74.7	89.4
<i>R</i> -factor to native (all reflections) (%)**		34.0	27.5	—
Refinement				
Starting <i>R</i> -factor (%)**		41.4	30.3	
<i>R</i> -factor after rigid body refinement (%)		33.7	27.0	

$$*R_{\text{sym}} = \frac{\sum_{hkl} |I_{hkl} - \bar{I}_{hkl}|}{\sum_{hkl} I_{hkl}}$$

$$**R_{\text{native}} = \frac{\sum |F_2 - F_1|}{\sum F_1}$$

two distinct sites in the A-B interface. Since cholera toxin as well as its A subunit have been reported to bind calcium [11] and since Ca^{2+} sites can often be occupied by lanthanides, due to a similarity of the ionic radius [23], it is quite possible that the observed samarium binding gives an indication of calcium sites. Calcium soaking of LT crystals up to a concentration of 15 mM CaCl_2 did not cause a change in cell dimensions and it can be concluded that calcium did not cause the same conformational change in the crystals as the lanthanides Er, Sm and Gd. Weak calcium binding at one or both lanthanide sites in LT and in the equivalent 'second site' in CT is, however, still possible.

A calcium ionophore effect of the cholera toxin or its subunits has been reported several times [9-12]. This effect can be inhibited very strongly by lanthanum ions ($K_i = 10^{-13}$). If, in fact, the calcium ionophore effect of CT and LT is caused by direct binding to the toxin this would fit well with the effects of lanthanide binding described in this paper, where we assume that La^{3+} has an affinity constant in the same range as the lanthanides tested here. The lanthanide-induced conformational change of A relative to B and the concomitant ion-mediated linkage between A and B subunits could be the cause of ionophore inhibition with this type of ion, e.g. by interference with a still unknown membrane modification process which is underlying the ionophoric properties of these toxins.

It is obvious that definite determination of calcium binding sites requires further investigations. Our results, however, leave no doubt about an intriguing position of lanthanide ions in LT and by implication for one of these also in CT. In addition the samarium binding induced a 2.3° rotation of the A subunit with respect to B, which is remarkable, especially since the soaking experiments took place in the crystalline state.

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